THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 246, No. 4, Issue of February 25, pp. 1107–1116, 1971
Printed in U.S.A.

Pyridine Nucleotide Metabolism in *Escherichia coli*

I. EXPONENTIAL GROWTH*

(Received for publication, July 20, 1970)

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SUMMARY

The pyridine nucleotide pool of *Escherichia coli* is made up almost exclusively of DPN and TPN. In an exponentially growing glucose culture an average cell contains 1,460,000 molecules of DPN (30% reduced) and 440,000 molecules of TPN (57% reduced). If the total volume of the cell were accessible to pyridine nucleotide, this would mean a total DPN concentration of 1.3×10^{-3} M and a TPN concentration of 3.9×10^{-4} M.

During exponential growth, in an average cell, 550 DPN molecules are being synthesized from external niacin, 270 DPN molecules are being transformed to TPN, and 140 TPN molecules are being broken down (to DPN) each second.

The pyridine nucleotide coenzymes DPN and TPN both have the nicotinamide ring as a prosthetic group for oxidation-reduction reactions. Despite their apparent chemical equivalence, the biological roles of these two coenzymes nevertheless appear to be specifically delineated. Generally, DPN is the cofactor for energy-yielding oxidations in the cell, while TPN, which is reduced mainly through the pentose-phosphate pathway serves as the source of biosynthetic reducing power. The existence of both coenzymes in all living cells is presumably required for independently regulating the oxidized and reduced levels of each nucleotide, and consequently the energy-yielding and the biosynthetic processes in the cell. Since these two coenzymes play key roles in cellular metabolism, it is reasonable to expect that their synthesis and breakdown is carefully regulated (for a recent review on pyridine nucleotides, see Reference 1).

In this paper, the kinetics of pyridine nucleotide metabolism in the bacterium *Escherichia coli* during exponential growth is investigated. We seek to establish the intracellular concentrations of the pyridine nucleotides and to understand how these concentrations are maintained. Our major finding is that the ratio of TPN:DPN is maintained because of a steady state balance between the synthesis of DPN (*de novo* or from external niacin), the synthesis of TPN from DPN, and the breakdown of TPN to DPN. The rates *in vivo* of these three reactions during exponential growth have been determined.

* This investigation was supported by Grant GB8610 from the National Science Foundation, and by an unrestricted research award from Eli Lilly and Company, Indianapolis, Indiana.

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EXPERIMENTAL PROCEDURE

Materials—^{14}C-Nicotinic acid was purchased from Amersham-Searle Corporation, Des Plaines, Illinois, and New England Nuclear. ^{3}H-Nicotinic acid was obtained from Amersham-Searle. Unless otherwise specified, all biochemicals were obtained from Sigma.

It was found that some batches of ^{14}C-niacin contained a growth inhibitor and an additional purification step was required to separate the niacin from this inhibitor. ^{14}C-Niacin (1 μmole) was dissolved in 0.1 ml of sterile water and spotted as a streak 4 cm wide on a thin layer chromatography cellulose plate. The chromatogram was developed with the following solvent: tertiary butyl alcohol, 40%; methyl ethyl ketone, 30%; water, 20%; concentrated NH_4OH, 10%. After developing and drying, the niacin spot was eluted with 3 ml of sterile water.

DEAE paper (Whatman DE 81) was purchased from H. Reeve Angel Company, New York, New York, and membrane filters (Bac-T-Flex nitrocellulose, type B-6) came from Schleicher and Schuell, Keene, New Hampshire.

Bacterial Strains—Most of the studies that are described in this paper were done with a niacin-requiring derivative of *Escherichia coli* 15T$^-$ (strain 555-7) which requires thymine, arginine, methionine, tryptophan, and niacin for growth. This strain was a gift of Dr. Cynthia Lark and will be referred to in this paper as *E. coli* 15T$^-$nic$^-$. Two other niacin-requiring strains of *Escherichia coli* K-12 were used for many of the experiments: Hfr No. 126, a nic C$^-$ strain, lacking quinolinate phosphoribosyl transferase and excreting quinolinic acid into the medium; P4X SB-16, a nic B$^-$, met$^-$, and B_1^- strain. An additional unmapped niacin-requiring mutant of *E. coli*, ATCC9723b, which is not a K-12 strain but behaves like a nic A$^-$ mutant in mixed extracts was also used. The first two strains support growth of T4 phage, while ATCC9723b does not; the three strains will be referred to as nic C, nic B, and nic A, respectively (2). These strains were the generous gift of Doctors Jerry L. R. Chandler and R. K. Gholson. In the early stages of this work, two other niacin-requiring strains, *Escherichia coli* AB3606 and AB3607 (a gift of Dr. E. Adelberg, Department of Microbiology, Yale University) were used.

Growth of Cultures—*E. coli* 15T$^-$nic$^-$ was generally grown in an M9 minimal salts medium (3) containing, per liter, 7 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, and 0.5 g of NaCl, and 0.4% glucose, 0.01 M $MgSO_4$, 0.001 M $CaCl_2$, 0.38 mg per ml of

L-arginine, 0.30 mg per ml of L-methionine, 0.14 mg per ml of L-tryptophan, 4 μg per ml of thymine, and 1 μg per ml of ^{14}C-niacin, unless otherwise specified. Growth was followed by measuring the absorbance at 595 mu, and by taking 0.1-ml aliquots at various times, diluting to 25 or 50 ml and counting with an electronic (Coulter) counter.

The nic A, nic B, and nic C strains were grown in the same medium except that thymine and the amino acids were omitted. The nic B media were supplemented with 60 μg per ml of methionine and 2 μg per ml of thiamine.

Measurement of Bacterial Volume—Cells were immobilized either by adding 1 volume of ethanol or by gentle heat. Photomicrographs were taken with a Zeiss photomicroscope on ADOX-KB14 film. Volumes were estimated from measurements on cells that were well focused on positive prints at a magnification of × 3000. Exponentially growing cells were assumed to be cylindrical with two half-spheres at both ends of the cylinder. Measurements were then taken of the length and width of the cylindrical portion of the cell and of the radii of the hemispheric ends. It was found that the ethanol-treated cells were 30 to 40% smaller than the heat-treated cells. In calculations of bacterial volume, only heat-treated preparations were considered. We are indebted to Dr. David Wolstenholme for advice regarding these measurements, and for taking photographs of the bacteria.

Measurement of Radioactivity Taken up by Cell—For measurement of total uptake of radioactive niacin, 0.1-ml aliquots of culture medium were added to 1 ml of M9 salts + 40 μg per ml of niacin, filtered on a membrane filter (Bact-T-Flex B6, Schleicher and Schuell), and washed with more M9 medium containing 40 μg per ml of niacin. The membrane filters were allowed to dry at room temperature and then counted in a scintillation counter.

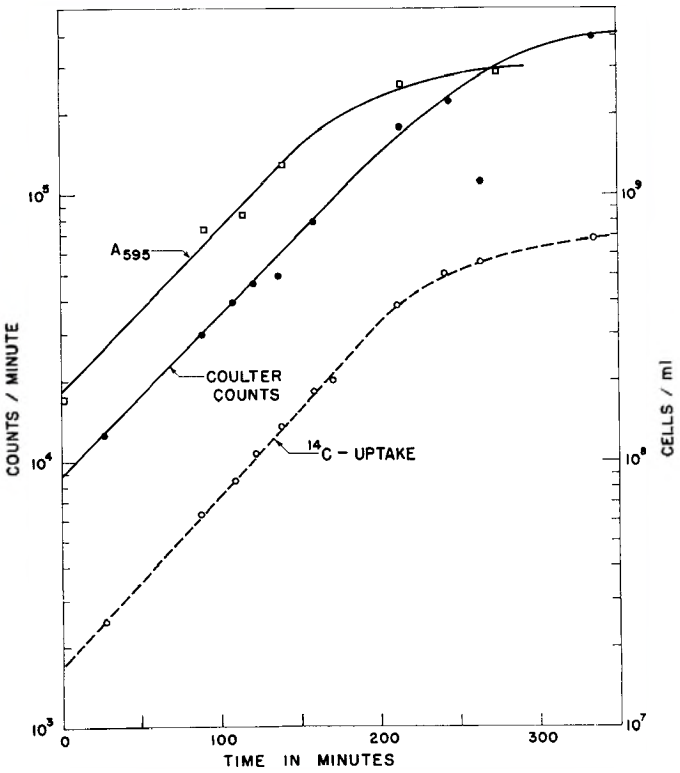

FIG. 1. Growth and uptake of ^{14}C-niacin by *E. coli* 15 T⁻nic⁻. An M9 salts medium (10 ml) containing 1 μg per ml of ^{14}C-niacin (59.1 Ci per mole) was inoculated with 0.1 ml of an overnight culture of *E. coli* 15 T⁻nic⁻. At the time intervals indicated, the absorbance at 595 mμ, cell counts and uptake of ^{14}C-niacin were determined (see "Experimental Procedure").

Preparation of Cell Extracts for Chromatographic Analysis—Extracts were generally prepared by taking a sufficient aliquot of cells to give approximately 10,000 cpm. The cells were collected by centrifugation at 7500 rpm for 10 min in a refrigerated RC2-B Sorvall centrifuge with an SS34 rotor, resuspended in an equal volume of ice-cold M9 salts medium (containing 40 μg per ml of niacin and no glucose), and collected again by centrifugation. The pellet was resuspended in 0.5 ml of 0.01 M Tris, pH 7.5, 0.001 M EDTA, and 0.005 M β-mercaptoethanol, sonically treated in a Raytheon sonicator for six 1-min periods at 0°, and brought to pH 4.5 with 0.2 M HCl. The extract was clarified by centrifugation and reneutralized to a pH between 6.5 and 7.5 with 0.2 M NaOH. In some experiments, the sonic extract was adjusted to pH 2 (instead of pH 4.5) and reneutralized with 0.1 M Tris, pH 9.7, (instead of 0.2 M NaOH).

When the reduced pyridine nucleotides were to be determined, 2.0 ml of culture was added directly to 1.0 ml of 0.33 M NaOH. The mixture was heated to 50° for 10 min, cooled, cautiously neutralized with cold 1 M HCl, and then directly chromatographed.

Chromatography of Cell Extracts—Chromatography was performed with DEAE paper. A 0.3-ml aliquot of the cell extract to be analyzed was mixed with 50 μl of a solution containing 2

TABLE I

Uptake of niacin by niacin-requiring strains of E. coli

These data are a summary of experiments similar to the one shown on Fig. 1 for four different niacin-requiring strains of *E. coli*. In all cases, the strains were grown in a glucose medium containing 1 μg per ml of ^{14}C-niacin (specific activity 59.1 Ci per mole). The data for exponentially growing cultures are averages of several experiments; the standard error of the mean is given. The data for stationary cells are based on single experiments.

Strain	^{14}C-Niacin uptake	
	Exponential	Stationary
	molecules/cell	
15T⁻ nic⁻	1.90 ± 0.10 × 10⁶	1.6 × 10⁶
nic A	1.89 ± 0.03 × 10⁶	1.6 × 10⁶
nic B	1.92 ± 0.07 × 10⁶	2.5 × 10⁶
nic C	1.92 ± 0.16 × 10⁶	1.6 × 10⁶

TABLE II

Niacin uptake as function of external niacin concentration

E. coli 15 T⁻ nic⁻ was grown in an M9 glucose medium containing thymine and amino acids, with the specified amounts of ^{14}C-niacin added (specific activity used, 20 to 60 Ci per mole). The uptake of radioactivity and the increase in the number of cells was followed as described under "Experimental Procedure."

External niacin concentration	^{14}C-Niacin uptake	Generation time
μg/ml	*molecules/cell*	*min*
0.02	0.040 × 10⁶	121
0.085	0.15 × 10⁶	67
0.10	0.32 × 10⁶	47
1.0	1.9 × 10⁶	40
24.3	1.9 × 10⁶	39

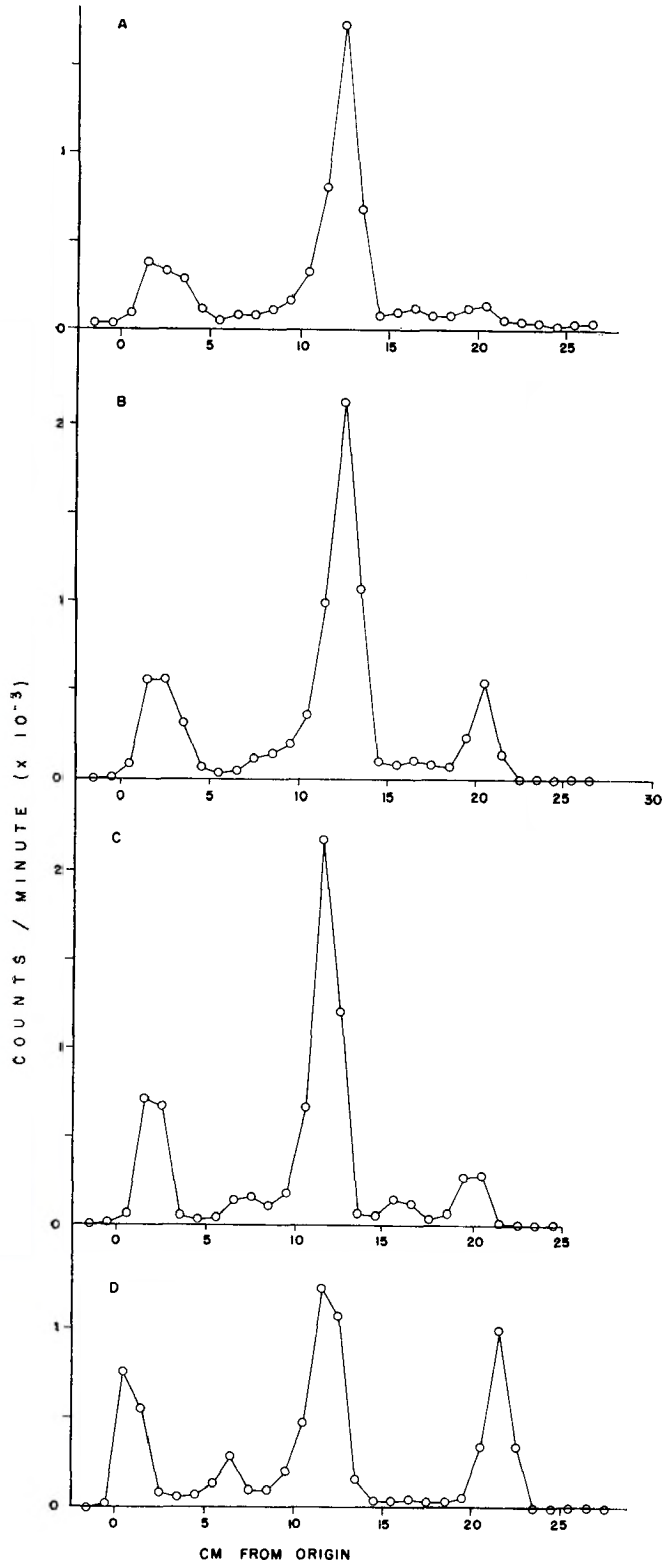

FIG. 2. Chromatography of intracellular pyridine nucleotides. Sonic extracts of *E. coli* 15 T⁻nic⁻ harvested in exponential growth in an M9-glucose culture containing ^{14}C-niacin were chromatographed on DEAE paper with 0.25 M NH_4HCO_3 as the developing solvent (see "Experimental Procedure"). In these chromatograms, the TPN peak is nearest the origin, DPN travels 12 to 13 cm, and nicotinamide travels 21 to 22 cm. N_aMN and N_aAD travel between DPN and TPN. Each chromatogram represents a different bacterial extract. Samples *A* and *B* were brought to pH 2 after sonic disruption with 0.2 M HCl, and neutralized with 0.1 M Tris base. Sample *C* was brought to pH 4.5 with 0.2 M HCl, and neutralized with 0.2 M NaOH. Sample *D* was not acidified after sonic disruption; the sonic extract was spotted directly on the chromatogram. Chromatograms *A*, *B*, *C*, and *D* yield analysis 1, 3, 4, and 6, respectively, on Table III. The extra peak in *D* is nicotinic acid mononucleotide.

mg per ml of DPN, 2 mg per ml of TPN, and 3.5 mg per ml of nicotinamide. The mixture was then spotted on a strip 2 cm wide at the origin, and chromatographed for 4 to 6 hours with 0.25 M NH_4HCO_3 as the solvent. The chromatogram was dried overnight, and the carrier spots were visualized under ultraviolet light and marked out with a pencil. Strips, 1 × 4 cm, were cut for determination of radioactivity.

RESULTS

Pyridine Nucleotide Content of Niacin-requiring Strains of E. coli—The level of niacin derivatives per bacterial cell was determined by growing niacin-requiring *E. coli* in a medium containing ^{14}C-niacin, and determining the uptake of radioactivity and the increase in cell number at various times. The experimental results for *E. coli* 15 T⁻nic⁻ are shown in Fig. 1. It is seen that there is a parallel increase in ^{14}C-niacin uptake, in cell number, and in the absorbance during the period of exponential growth. From the specific activity of the ^{14}C-niacin, and the ratio between niacin uptake and cell number at any time, it is possible to determine the total number of molecules of pyridine nucleotide per bacterium. From the data in Fig. 1, a value of 1.90×10^6 molecules of niacin taken up per cell is obtained. The results for four different niacin-requiring mutants of *E. coli* are tabulated on Table I. The four mutants yield similar values for the number of niacin molecules per cell in both the exponential and stationary stages of growth. These mutants were independently isolated, and it is known that the mutations in two of them (nic B and nic C) map at different sites (2). The nic C mutant is defective in quinolinate phosphoribosyl transferase and excretes quinolinic acid into the medium; there is no pathway *de novo* for pyridine nucleotide biosynthesis known which does not involve quinolinic acid. Thus, these results suggest that the value of 1.90×10^6 represents the total niacin content of these cells, and that all four mutants are unable to synthesize any niacin. More rigorous evidence for this assertion will be presented elsewhere.[1]

Uptake of Niacin as Function of External Niacin Concentration—The data summarized in Table I give the intracellular pyridine nucleotide levels during exponential growth in a medium containing one particular concentration of ^{14}C-niacin (1 μg per ml). This level is sufficient to maintain the generation time characteristic of the prototrophic parent strain in a glucose medium (40 min at 37°). The change in the number of pyridine nucleotide molecules per bacterial cell as a function of the external niacin concentration has been determined by repeating the experiment in Fig. 1 with different concentrations of niacin in the medium (Table II). It is seen that the value of 1.90×10^6 molecules per cell remains constant even if the external niacin concentration is increased 25-fold. On the other hand, if the niacin concentration is decreased until the generation time increases significantly, then the levels of pyridine nucleotide per cell are found to be below normal. Thus, a level of 1.90×10^6

[1] J. McLaren and B. M. Olivera, manuscript in preparation.

TABLE III

Chromatography of pyridine nucleotides

Treatment of extracts. Extracts of the indicated niacin-requiring strains of *E. coli* which had been growing in a medium containing 1 μg per ml of ^{14}C-niacin were prepared by sonic disruption as described under "Experimental Procedure." The extract was brought to the acid pH indicated with 0.2 M HCl and reneutralized with either 0.1 M Tris base or 0.2 M NaOH. An appropriate aliquot of the extract was used for chromatography on DEAE paper with 0.25 M NH_4HCO_3 as solvent except for Run No. 2, in which 0.15 M NH_4HCO_3-0.05 M NH_4Cl, pH 6.8, was used. The fractions of the total radioactivity found in DPN, TPN, and nicotinamide are tabulated.

Run No.	pH acidified to	Neutralized with	Strain	Distribution		
				TPN	DPN	Nicotinamide
				% total radioactivity		
			Exponential			
1	2.0	Tris	15 T⁻ nic⁻	19	67	4
2	2.0	Tris	15 T⁻ nic⁻	21	70	4
3	2.0	Tris	15 T⁻ nic⁻	20	58	12
4	Not acidified		15 T⁻ nic⁻	20	45	25
5	4.5	Tris	15 T⁻ nic⁻	19	63	7
6	4.5	NaOH	15 T⁻ nic⁻	21	60	9
7	4.5	NaOH	15 T⁻ nic⁻	20	55	14
8	4.5	NaOH	15 T⁻ nic⁻	15	60	9
9	4.5	NaOH	15 T⁻ nic⁻	13	44	32
10	4.5	NaOH	nic A	15	60	12
11	4.5	NaOH	nic B	22	57	7
12	4.5	NaOH	nic B	21	56	2
13	4.5	NaOH	nic C	15	58	8
			Stationary			
14	4.5	NaOH	15 T⁻ nic⁻	24	51	6
15	4.5	NaOH	nic A	22	62	7
16	4.5	NaOH	nic B	25	49	5
17	4.5	NaOH	nic B	15	51	10
18	4.5	NaOH	nic C	19	56	5

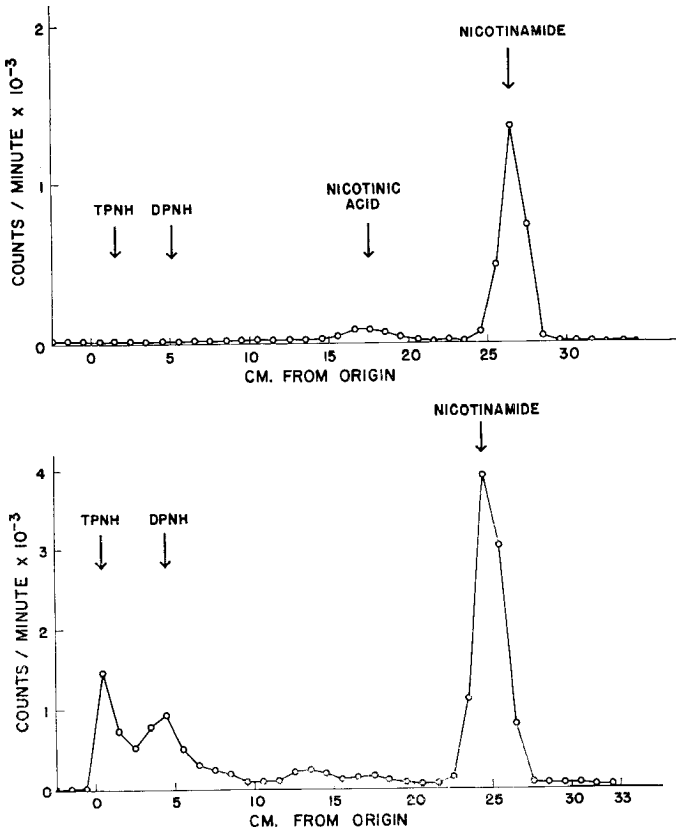

FIG. 3. Analysis of reduced pyridine nucleotides. The *top figure* was prepared by sedimenting and washing cells as described under "Experimental Procedure," and then adding 0.33 M NaOH to the washed cells, heating at 50° for 10 min and neutralizing with cold 1 M HCl. In the *bottom figure*, the cells which had been growing for many generations in a medium containing 1 μg per ml of ^{14}C-niacin were transferred by filtration on a millipore filter to an identical medium containing 1 μg per ml of unlabeled niacin (so that no extracellular label remained). After 40 min, 1 ml of 0.33 M NaOH was added directly to 2 ml of medium, and the sample was then treated as described above.

molecules per cell is characteristic of normal growth of niacin auxotrophs of *E. coli* in this medium.

Metabolic Fate of Niacin during Exponential Growth—We have investigated the fate of the ^{14}C-niacin, once it is taken up by the bacterial cell. Chromatograms of the intracellular ^{14}C label from cells in exponential growth are shown in Fig. 2. The label is present predominantly as DPN and TPN. A substantial amount of nicotinamide is usually found; however, the amount which appears on chromatography is very variable as is shown in Fig. 2 and Table III, and can vary from 2 to 32%. We believe that the nicotinamide found on chromatography results from hydrolysis of DPN and TPN after the cells are lysed, probably through the action of a DPNase (or TPNase) and by chemical hydrolysis.

We find that in preparing extracts, the following conditions are necessary to keep the amount of nicotinamide on the chromatograms below 10%: (*a*) the extracts must not stand any more than necessary after sonic disruption, even if kept in an ice bucket; (*b*) the acidification step must not be omitted (see Fig. 2*D*), and (*c*) the NaOH must be chilled and added very slowly to the extract during neutralization. We have also found that acidification to pH 2 (instead of pH 4.5), reneutralization with 0.1 M Tris, pH 9.7 (instead of 0.2 M NaOH), or chromatography in 0.15 M NH_4HCO_3-0.05 M NH_4Cl, pH 6.8, help to decrease the nicotinamide levels to less than 5% and increase the over-all recovery as DPN and TPN. However, with all these precautions, the amount of nicotinamide found is still variable (compare Fig. 2, *A* to *B*). In addition, acidification to pH 2 and with 0.15 M NH_4HCO_3-0.05 M NH_4Cl, pH 6.8, as the chromatographic solvent result in poorer chromatographic separation and broader peaks.

We conclude from our data that essentially all the pyridine nucleotide in the cell is present as DPN and TPN, and that the TPN:DPN ratio = 0.30. In favorable cases, over 90% of the radioactivity is in fact recovered as DPN and TPN, and about 95% as DPN, TPN, and nicotinamide. Since it is most likely that all the nicotinamide found is formed by hydrolysis after lysis, then at least 95% of the intracellular pyridine nucleotide is DPN and TPN. The identity or origin of the remainder of the radioactivity is uncertain; it is apparently a mixture of other compounds including niacin, NMN, and N_aMN[2] and whether

[2] The abbreviations used are: N_aMN, nicotinic acid mononucleotide; N_aAD, nicotinic acid adenine dinucleotide.

TABLE IV

Fraction of pyridine nucleotides in oxidized and reduced form

Treatment	Distribution (unless otherwise specified, as percentage of total pyridine nucleotide)			
	TPN		DPN	
	Oxidized	Reduced	Oxidized	Reduced
	%	%	%	%
Cells washed, sedimented and sonically treated	23		77	
Cells treated with alkali directly[a]		13		23
Probable intracellular distribution[b]	10	13	54	23
Number of molecules per cell	190,000	250,000	1,000,000	440,000
Intracellular molarity[c]	1.7×10^{-4}	2.2×10^{-4}	9.0×10^{-4}	4.0×10^{-4}

[a] As shown on Fig. 3, most of the remainder of the radioactivity is nicotinamide. A small amount of nicotinic acid (6%) is also found which probably results from the hydrolysis of nicotinamide during the alkali treatment. The figures for reduced pyridine nucleotides have been corrected for the redistribution of label in DPN and TPN that takes place during a 40-min chase (see Figs. 5 and 6).

[b] It is assumed that the oxidized pyridine nucleotides recovered by the first treatment represent both the oxidized and reduced intracellular levels.

[c] These figures are calculated assuming that the entire volume of the cell is accessible to the pyridine nucleotides.

these are present in the cell in the amounts found in the chromatogram, or are a result of hydrolysis or chemical degradation after lysis cannot be determined. In any case, in the chromatograms in which the greatest precautions are taken to avoid hydrolysis, none of these compounds are present in amounts >3%. Chromatography of the pyridine nucleotide from nic A, B, and C mutants yields substantially the same distribution as found for *E. coli* 15T⁻nic⁻ (Table III).

Determination of Reduced Pyridine Nucleotides—Under the conditions for preparing cell extracts described in Fig. 2, the pyridine nucleotides are found exclusively in the oxidized form. No detectable DPNH or TPNH peaks are apparent on the chromatograms. We have found that sedimentation and washing the cells causes complete oxidation of DPNH and TPNH. The reduced pyridine nucleotides can be recovered only by adding alkali directly to the growing culture. This is illustrated in Fig. 3. It is unclear why this potent DPNH and TPNH oxidase activity is unmasked by sedimenting and washing the cells; however, the respiratory chain is probably not involved as an entire entity since the oxidation of DPNH and TPNH takes place even in the presence of cyanide. When alkali is added directly to the medium, the chromatographic pattern obtained is reproducible, and it is possible to calculate the fraction of the total label which is present as DPNH or TPNH. The total number of pyridine nucleotide molecules in all forms is shown in Table IV.

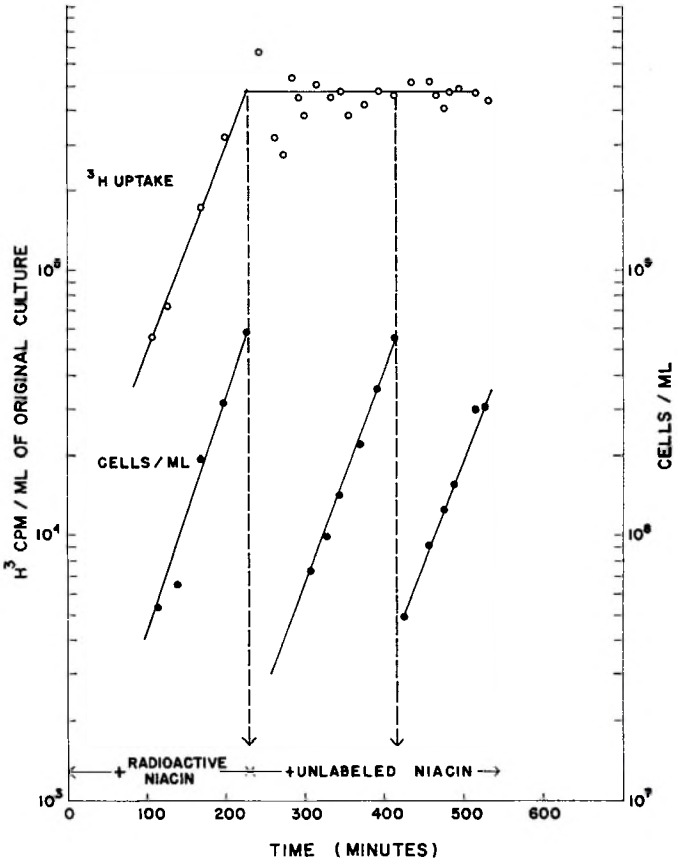

FIG. 4. Transfer of *E. coli* 15 T⁻nic⁻ from a medium containing ^{3}H-niacin to a medium containing unlabeled niacin. A culture of *E. coli* 15 T⁻nic⁻ was grown in an M9-glucose medium ("Experimental Procedure") containing 1 μg per ml of ^{3}H-niacin (specific activity, 787 Ci per mole). After 240 min of growth in this medium, 2 ml of the culture was filtered on a millipore filter and washed with M9-salts solution. The filter was resuspended in 40 ml of M9 culture medium containing 1 μg per ml of unlabeled niacin. At 415 min, 4 ml of this culture was filtered and resuspended in 40 ml of an identical medium. The ^{3}H-niacin uptake, coulter counts, and absorbance were followed as described under "Experimental Procedure." Samples were taken for chromatography at various times after transfer.

Intracellular Concentration of Pyridine Nucleotides—The intracellular concentration of each pyridine nucleotide is a parameter of some interest. We have measured the volume of *E. coli* 15T⁻nic⁻ growing exponentially and found this to be 1.9 ± 0.2 μ^3. (A description of the calculations used for determining the cell volume is given under "Experimental Procedure"). From this value, it is possible to calculate the concentrations of all pyridine nucleotides, assuming that they are evenly distributed throughout the cell. The results are tabulated in Table IV. The concentrations calculated are minimal values, since it is assumed in these calculations that all parts of the cell are accessible to the pyridine nucleotides.

Pulse-Chase Experiment in Exponentially Growing Culture—The radioactivity distribution of the pyridine nucleotides depends on whether the bacteria are grown continuously in the radioactive medium or given a pulse followed by a cold chase. Fig. 4 shows a pulse-chase experiment in which the cells are grown exponentially in the presence of ^{3}H-niacin of high specific activity

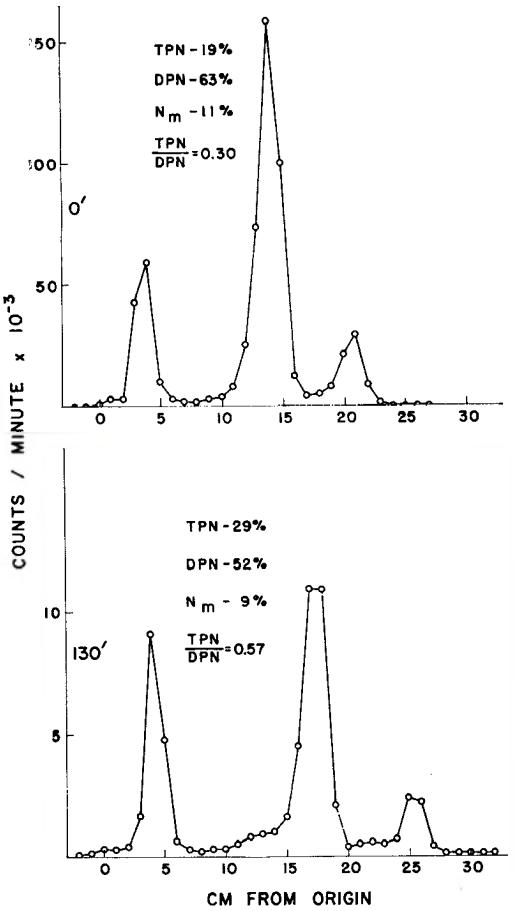

FIG. 5. ^{14}C distribution in pyridine nucleotides after a pulse, and after a pulse followed by a 130-min chase. *E. coli* 15 T⁻nic⁻ was grown in a medium containing 1 μg per ml of ^{14}C-niacin, and then transferred to a medium containing 1 μg per ml of unlabeled niacin. The figure shows the intracellular distribution of pyridine nucleotides at the time of transfer and after growth for 130 min in the second medium. Extracts were subjected to chromatography on DEAE paper ("Experimental Procedure"): Under these conditions, nicotinamide travels fastest, followed by DPN and TPN, respectively.

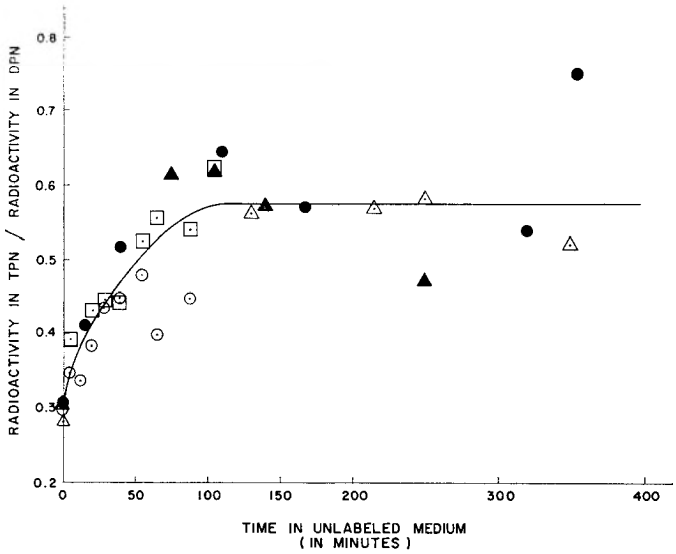

FIG. 6. The change in the ratio of radioactivity in TPN to radioactivity in DPN as a function of the chase time. The ratio of radioactivity in TPN relative to the radioactivity in DPN is plotted as a function of time after transfer to the unlabeled medium. The data in this figure are derived from experiments of the type shown in Figs. 4 and 5. The different *symbols* represent different bacterial cultures. A source of uncertainty in the TPN: DPN ratio is the presence of a variable amount of nicotinamide in different chromatograms.

and then washed and diluted into a medium containing unlabeled niacin. In this way, the cells are maintained in the exponential phase of growth, the external niacin concentration does not change, and the distribution of the radioactive niacin which was pulsed in can be followed at various times after the shift to the unlabeled medium.

There are two noteworthy experimental findings: (*a*) the intracellular label is essentially conserved through seven generations of exponential growth (Fig. 4) and (*b*) the TPN:DPN ratio of labeled nucleotide increases slowly during the chase and reaches an apparent equilibrium value of 0.57 (Figs. 5 and 6). This value is attained about two generations after transfer to the unlabeled medium and remains constant thereafter. Thus, although for the total pyridine nucleotide, the TPN:DPN ratio is 0.30, the ratio of radioactivity in TPN to radioactivity in DPN increases to 0.57 after a long chase.

DISCUSSION

The values we have obtained for the intracellular levels of the different pyridine nucleotides are in reasonable agreement with previous studies (although for direct comparisons, certain assumptions regarding wet and dry cell mass must be made) (4–6). However, we find a TPN:DPN ratio significantly higher than most values previously reported. The difference is unexplained, although we think it quite unlikely that any artifact of preparation would cause a conversion of DPN into TPN.

These studies also indicate the careful control that the cell exercises over niacin metabolism. Thus, unlike the lack of control of riboflavin metabolism (7) there is an upper limit in the amount of pyridine nucleotide a cell may synthesize. The data suggest that once this threshold limit is reached, a stringent control mechanism prevents a further increase in the pyridine nucleotide levels even with large increases in the external niacin concentration. The studies of Imsande and Pardee (8) indicate that control may be exerted by repression of N_aMN pyrophosphorylase. At high concentrations of niacin in the medium, the levels of this enzyme may fall such that the rate of synthesis of pyridine nucleotides does not exceed the threshold value.

The concentrations of oxidized DPN (9×10^{-4}), DPNH (4×10^{-4}), oxidized TPN (1.7×10^{-4} M), and TPNH (2.2×10^{-4}) which are obtained are above the K_m values for most DPN- or TPN-requiring enzymes. This suggests that such enzymes are close to saturation with respect to pyridine nucleotides during exponential growth in a glucose medium containing 1 μg per ml of niacin. In these experiments, we find most of the DPN in an exponentially growing culture in the oxidized form and most of the TPN in the reduced form. The experiments of Wright and Sanwal (9) yield somewhat different results with respect to the DPNH:DPN ratio in glucose-grown cells.

In *E. coli* the pyridine nucleotides are synthesized through the Preiss-Handler pathway (see Reference 8). Since cells growing exponentially contain all the intracellular niacin as DPN and TPN, the intermediate compounds in DPN biosynthesis (N_aMN

and N_aAD) must be present in negligible amounts under these conditions. Intracellular niacin is remarkably conserved. After a pulse of radioactive niacin followed by a cold chase during which the cells increase in number 100-fold, no measurable radioactivity is excreted, and DPN and TPN are the only metabolites with a significant amount of label. This indicates that the niacin moiety of any molecule of DPN and TPN breaking down in the cell has a 100% probability of being eventually recycled back to DPN or TPN.

The metabolism of niacin in *E. coli* is therefore somewhat unusual because the niacin ring has no metabolic fate other than to be ultimately transformed to DPN and TPN.

During exponential growth, the pyridine nucleotides may be represented as being in a steady state as is shown in the figure below.

$$\text{Niacin} \xrightarrow[\text{cell wall}]{R_u} \text{DPN} \underset{R_2}{\overset{R_1}{\rightleftharpoons}} \textit{TPN}$$

Where R_u is the rate of formation of DPN from external niacin in each cell (Intermediate metabolites are present in such small amounts that their accumulation can be neglected. As far as is known, TPN can only be synthesized from DPN; thus, R_u is essentially equal to the total uptake of niacin from the medium.), R_1 is the average rate of conversion of DPN to TPN per cell, and R_2 is the average rate of breakdown of TPN to DPN per cell (We include here the breakdown into any compound which is eventually transformed back to DPN before it becomes TPN. Since all the pyridine ring is conserved during exponential growth, and TPN is only made from DPN, then R_2 represents the total breakdown of TPN).

Under our experimental conditions, the cells are growing exponentially with a generation time τ of 2400 sec, and the average DPN content per cell is 1.46×10^6 DPN molecules per cell and the TPN content is 0.437×10^6 molecules per cell. It follows that during balanced growth:

$$R_u + R_2 - R_1 = \text{rate of DPN accumulation per cell} = \frac{1.46 \times 10^6 \times 0.693}{\tau} \quad (1a)$$

$$= 422 \text{ molecules per sec per cell}$$

$$R_1 - R_2 = \text{rate of TPN accumulation per cell} = \frac{4.37 \times 10^5 \times 0.693}{\tau} \quad (1b)$$

$$= 126 \text{ molecules per sec per cell}$$

(Equations 1*a* and 1*b* are rigorously derived in the appendix).

We have the interesting situation where upon transfer of the culture to the unlabeled medium, the ratio of labeled TPN: labeled DPN increases from 0.30 to 0.57 after two generations of growth. A simple explanation is provided by the flow chart above. The *total* DPN level is determined by the relative rates R_u, R_2, and R_1 as shown on Equation 1*a*. In contrast, after a shift to the unlabeled niacin medium, the radioactive DPN level would be determined initially only by the relative rates of R_2 and R_1, since none of the DPN molecules being formed through the uptake route (R_u) are radioactive. However, we know that R_1 is greater than R_2, and thus there will be an initial fall in the total radioactivity in DPN.

This will result in a lower specific activity of DPN compared to TPN. Indeed, after about two generations, when a steady state is reached with respect to the distribution of the label, the ratio of total TPN:total DPN = 0.30, but the ratio of labeled TPN:labeled DPN = 0.57. The equilibrium ratio of labeled TPN:labeled DPN would only be determined by the DPN-TPN interconversion (since these are the only two compounds that have any significant radioactivity). Thus, it is possible from the data to extract quantitative information about the kinetics of DPN and TPN interconversion.

When the radioactivity distribution reaches equilibrium, then:

$$\text{Rate of conversion of radioactive DPN to TPN} = \text{rate of conversion of radioactive TPN to DPN} \quad (2a)$$

If S_{DPN} = the specific activity of DPN, and S_{TPN} = the specific activity of TPN, then Equation 2*a* may be written

$$S_{DPN} \times R_1 = S_{TPN} \times R_2 \quad (2b)$$

Now

$$S_{DPN} = \frac{\text{radioactivity in DPN}}{\text{total DPN concentration}} = \frac{\text{cpm}_{DPN}}{(DPN)} \quad (3a)$$

and

$$S_{TPN} = \frac{\text{radioactivity in TPN}}{\text{total TPN concentration}} = \frac{\text{cpm}_{TPN}}{(TPN)} \quad (3b)$$

However, our experimental result is that at equilibrium during exponential growth

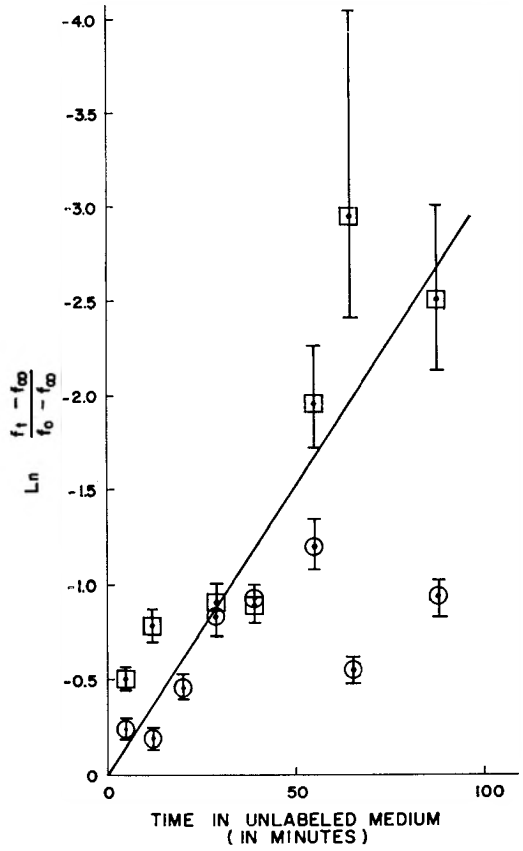

FIG. 7. The data of Fig. 6 are plotted in the form of Equation 6*a* (see text). The *error bars* represent the uncertainty in the abscissa with an error of 0.01 in the TPN:DPN ratio.

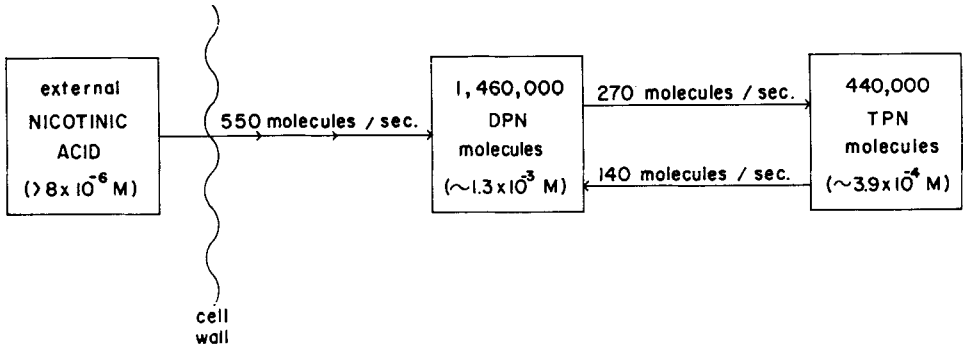

FIG. 8. A steady state representation of DPN and TPN formation in *E. coli* 15 T⁻nic⁻ growing exponentially.

$$\frac{\text{cpm}_{\text{TPN}}}{\text{cpm}_{\text{DPN}}} = 0.57 \tag{4}$$

$$\frac{(\text{TPN})}{(\text{DPN})} = 0.30 \tag{5}$$

Combining Equations 3, 4, and 5, we find that

$$\frac{S_{\text{TPN}}}{S_{\text{DPN}}} = \frac{\dfrac{\text{cpm}_{\text{TPN}}}{(\text{TPN})}}{\dfrac{\text{cpm}_{\text{DPN}}}{(\text{DPN})}} = \frac{\dfrac{\text{cpm}_{\text{TPN}}}{\text{cpm}_{\text{DPN}}}}{\dfrac{(\text{TPN})}{(\text{DPN})}} = \frac{0.57}{0.30} = 1.90$$

It follows from Equations 1*b* and 2*b* that

$$\frac{S_{\text{TPN}}}{S_{\text{DPN}}} = \frac{R_1}{R_2} = 1.90$$

$$R_1 - R_2 = 1.90\,R_2 - R_2 = 0.90\,R_2$$

= 126 molecules per sec per cell

$$R_2 = \frac{126}{0.90} = 140 \text{ molecules per sec per cell,}$$

rate of breakdown of TPN

R_1 = 266 molecules per sec per cell,

actual rate of synthesis of TPN

It should be mentioned that an alternative explanation for a shift in the ratio $\text{cpm}_{\text{TPN}}:\text{cpm}_{\text{DPN}}$ during a pulse-chase experiment is the presence of more than one intracellular pool of pyridine nucleotides. It is possible, for example, that there is an "outer" pool containing only DPN, and an "inner" pool containing TPN and DPN at a ratio of 0.57. If DPN moves from the outer pool to the inner pool irreversibly, then in a pulse-chase experiment, an increase in the TPN:DPN radioactivity ratio from the ratio for the total cell, to that of the inner pool would be observed.

However, possibilities of this sort have been virtually eliminated because the steady state hypothesis above makes a very stringent prediction about the rate of change in the radioactivity distribution of the pyridine nucleotides during the chase period. If the amount of radioactivity in DPN and TPN is determined only by R_u, R_1, and R_2, and the R_u contribution is cut off during the chase period, then the distribution will change at a rate dependent on the magnitude of R_1 and R_2. Thus if the steady state hypothesis were true, an approach to equilibrium calculation should yield values of R_1 and R_2 identical with those calculated from the final equilibrium distribution of the radioactivity during the chase. If on the other hand the redistribution of radioactivity were caused by two separate pools of pyridine nucleotides, then the approach to the new distribution would not be expected to follow these predictions.

As shown in the Appendix the steady state hypothesis predicts that the radioactivity distribution will change in the following way

$$\text{Ln}\,\frac{f_{\text{DPN},\,t} - f_{\text{DPN},\,t=\infty}}{f_{\text{DPN},\,t=0} - f_{\text{DPN},\,t=\infty}} = -At \tag{6a}$$

Where $f_{\text{DPN},t}$ is the fraction of the radioactivity found in DPN at time t, $f_{\text{DPN},t=0}$ is the fraction of radioactivity in DPN at the beginning of the chase and $f_{\text{DPN},t=\infty}$ is the final equilibrium fraction of radioactivity in DPN.

It can be shown that:

$$A = \frac{0.693}{\tau}\left[\frac{R_1}{R_u - R_1 + R_2} + \frac{R_2}{R_1 - R_2}\right] \tag{6b}$$

Since τ, $(R_u - R_1 + R_2)$, and $(R_1 - R_2)$ are all known quantities (see Equation 1), the values of R_1 and R_2 can be calculated from Equation *6b*.

The data from Fig. 6 are replotted in Fig. 7 to fit the form of Equation 6*a*. It is seen that the data do fit a straight line, and from the slope of the line we find that

$$A = 1/2280 = \frac{0.693}{\tau}\left[\frac{R_1}{R_u - R_1 + R_2} + \frac{R_2}{R_1 - R_2}\right]$$

By substituting the appropriate values for τ, $R_u - R_1 + R_2$ and $R_1 - R_2$ from Equations 1*a* and 1*b*, we find that $R_1 = 245$ and $R_2 = 119$.

These values are in very good agreement with the equilibrium calculation. We therefore conclude that during exponential growth the levels of DPN and TPN are maintained by a steady state balance between reactions R_u, R_1, and R_2. A representation of the flow of niacin in an average exponentially growing cell under our experimental conditions is shown in Fig. 8.

The considerations above lead to the prediction that if niacin-requiring cells were fasted for niacin, then there should be an immediate increase in the TPN:DPN ratio (because $R_u = 0$, and R_1 would initially be 1.9 R_2). As will be detailed in a following paper[3] this prediction is in fact borne out.

Finally, the experimental results indicate that the breakdown of TPN is one of the major processes which determines the relative levels of DPN and TPN in the cell. Although the

[3] R. Lundquist and B. M. Olivera, manuscript in preparation.

experiments above permit us to accurately estimate the rate at which TPN is breaking down under these growth conditions (140 molecules per sec per cell), they do not yield any information as to the actual mechanism of TPN breakdown. A TPN phosphatase or a TPNase are among the possible enzymes which may be involved. The regulation of the responsible enzyme would be of some interest since changes in the activity of such an enzyme could drastically alter the intracellular TPN:DPN ratio.

Acknowledgment—We wish to thank Professor Norman Davidson for his helpful suggestions regarding the kinetic treatment of the data.

APPENDIX

Calculations for Rate of Accumulation of DPN and TPN per Cell

Given an exponential culture, let n = number of cells at time t. Then:

$$\frac{dn}{dt} = Kn;\ n = n_0e^{kt} \tag{1}$$

where n_0 is the number of cells at $t = 0$. The doubling time, $\tau = 0.693/k$. If we consider the scheme

$$\text{Niacin} \xrightarrow{R_u} \text{DPN} \underset{R_2}{\overset{R_1}{\rightleftharpoons}} \text{TPN}$$

where R_u, R_1, and R_2 are the rates of each reaction in molecules per sec per cell. (We will assume that these rates are constants. It is possible that the bigger the cell, the faster the rate, but we can average over all cells in the exponential culture. The rate R_u may in fact be constant over all cells since it has been shown that uptake is generally independent of the size of the cells.)

We now define: Σ_D = total number of molecules of DPN in all cells at time t; Σ_T = total number of molecules of TPN in all cells at time t. It follows that:

$$\frac{d\Sigma_D}{dt} = nR_u - nR_1 + nR_2 \tag{2}$$

$$\frac{d\Sigma_T}{dt} = nR_1 - nR_2 \tag{3}$$

If we then substitute for n from Equation 1, we get

$$\frac{d\Sigma_D}{dt} = n_0e^{kt}(R_u - R_1 + R_2)$$

$$\Sigma_D = \frac{(R_u - R_1 + R_2)n_0e^{kt}}{K} = \frac{(R_u - R_1 + R_2)n}{K} \tag{4}$$

Thus

$$\frac{\Sigma_D}{n} = 1.46 \times 10^6 = \frac{(R_u - R_1 + R_2)\tau}{0.693}$$

Now, since $\tau = 40$ min $= 2400$ sec, then:

$$R_u - R_1 + R_2 = 422 \text{ molecules per sec per cell}$$

Similarly:

$$\frac{\Sigma_T}{n} = 4.37 \times 10^5 = \frac{(R_1 - R_2)\tau}{0.693} \tag{5}$$

$$R_1 - R_2 = 126 \text{ molecules per sec per cell}$$

Approach to Equilibrium Calculation for Pulse-chase Experiment

We define S_n, S_D, S_T, as the specific activity of niacin, DPN, and TPN, respectively.

$$\begin{aligned} S_D\Sigma_D &= \text{total radioactivity in DPN} \\ S_T\Sigma_T &= \text{total radioactivity in TPN} \end{aligned} \tag{6}$$

If the cells are shifted from radioactive niacin to unlabeled niacin, then

$$\frac{d(S_D\Sigma_D)}{dt} = nS_nR_u - nS_DR_1 + nS_TR_2 = -nS_DR_1 + nS_TR_2 \tag{7a}$$

(since during the cold chase, $S_n = 0$).

$$\frac{d(S_T\Sigma_T)}{dt} = nS_DR_1 - nS_TR_2 \tag{7b}$$

We know from Equation 4 that:

$$\Sigma_D = \frac{(R_u - R_1 + R_2)n}{K}$$

and from Equation 5 that:

$$\Sigma_T = \frac{(R_1 - R_2)n}{K}$$

Substituting for n in Equation 7a, we get

$$\frac{dS_D\Sigma_D}{dt} = \frac{-KR_1S_D\Sigma_D}{R_u - R_1 + R_2} + \frac{KS_T\Sigma_TR_2}{R_1 - R_2} \tag{8}$$

but $\Sigma_TS_T + \Sigma_DS_D = (\Sigma S)^0$, where $(\Sigma S)^0$ is the total radioactivity. Equation 8 then becomes

$$\frac{\frac{dS_D\Sigma_D}{dt}}{(\Sigma S)^0} = -\left[\frac{(S_D\Sigma_D)}{(\Sigma S)^0}\right] \times \left[\frac{R_1K}{R_u - R_1 + R_2} + \frac{R_2K}{R_1 - R_2}\right] + \frac{R_2K}{R_1 - R_2} \tag{9}$$

If we define:

$$A = \frac{R_1K}{R_u - R_1 + R_2} + \frac{R_2K}{R_1 - R_2} = \frac{0.693}{\tau}\left[\frac{R_1}{R_u - R_1 + R_2} + \frac{R_2}{R_1 - R_2}\right]$$

$$B = \frac{0.693\ R_2}{(R_1 - R_2)\tau}$$

$$f = \frac{S_D\Sigma_D}{(\Sigma S)^0} = \text{fraction of total radioactivity in DPN}$$

then Equation 9 is

$$\frac{df}{dt} = -Af + B \tag{10}$$

Solution is:

$$\frac{f_t - f_\infty}{f_0 - f_\infty} = e^{-At} \tag{11}$$

where f_t, f_0, f_∞ are the fraction of the radioactivity in DPN at times t, 0, and ∞, respectively. Then:

$$\text{Ln}\frac{f_t - f_\infty}{f_0 - f_\infty} = -At \quad (12)$$

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